EXHIBIT 1

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Glutathione Contents of Tissues in the Aging Mouse

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1. Previous results from this laboratory demonstrated that the erythrocyte content of reduced glutathione (GSH) decreased as a function of both increasing cell age and mouse age [Abraham, Taylor & Lang (1978) *Biochem. J.* 174, 819–825]. In the present investigation glutathione concentrations were determined in other tissues of the C57BL/6J mouse of different ages (6–31 months) throughout the life-span. 2. At all ages the total glutathione and the GSH concentrations in liver were 3 times that in kidney and 10 times that in heart. In the old (31 months) mouse the GSH contents were lower by 30% in the liver, 34% in the kidney and 20% in the heart than in the mature (17–23 months) animals. 3. The oxidized glutathione (GSSG) concentrations of the tissues did not vary with age and constituted less than 3% of the total glutathione. 4. The decreases in GSH concentrations were not due to changes in organ weights, which were constant from 10 to 36 months of age. 5. These findings extend our previous results and indicate that a general characteristic of aging tissues may be a decrease in GSH concentrations. Further, this is consistent with our hypothesis that the reducing potential of tissues decreases in senescence.

Glutathione is a ubiquitous cellular constituent that is the most abundant thiol reducing agent in mammalian tissues. It has been implicated in a wide variety of biological reactions, according to several reviews (Jocelyn, 1972; Flohé *et al.*, 1974; Meister & Tate, 1976; Arias & Jakoby, 1976). In spite of the biochemical evidence that glutathione functions in the maintenance of thiol groups of proteins, removal of H_2O_2, transport of amino acids and detoxification of foreign compounds, its physiological significance still requires elucidation.

A possible role of GSSG as a test of senescence has been proposed in a review (Oeriu, 1964). However, the evidence was inconclusive, because of relatively high GSSG values, which result from the rapid oxidation of GSH during processing and storage unless precautions are taken. Also in most cases immature animals were used, and thus the reported changes reflected growth rather than aging or senescence processes. In other instances the biological ages of the animals determined by survival curves or median survival times of the populations were not stated. However, there is other evidence of decreases in GSH concentrations, but only in certain specialized tissues, e.g. human lens (Harding, 1970) and aging erythrocytes (Waller *et al.*, 1974).

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

Our previous results demonstrated a lower glutathione status in erythrocytes of aging C57BL/6J mice (Abraham *et al.*, 1978). Decreases occurred in free thiol groups, GSH concentration and GSSG reductase activity as a function of both erythrocyte age and mouse age. These findings extended our earlier related evidence of marked aging-specific decreases in $NADP^+$-linked enzyme activities in the mouse and mosquito and in $NADPH/NADP^+$ ratios in the mosquito (Stephan *et al.*, 1966; Lang & Acree, 1967; Lang & Stephan, 1967).

A decrease in GSH content of aging tissues has not been established as a general phenomenon. To this end the aim of this investigation was to determine the concentrations of GSH and GSSG in liver, kidney and heart tissues of C57BL/6J mice of different ages throughout their life-span, including senescence. A preliminary account of this work has been presented (Hazelton & Lang, 1978*a*).

Materials and Methods

Animals

The C57BL/6J mice in these studies were purchased from the Jackson Memorial Laboratories, Bar Harbor, ME, U.S.A. Both male weanlings and retired breeders (8–9 months old) were maintained in our aging colony and used for the 6-month-old and 10–36-month-old age groups, respectively. All

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animals were housed in groups of four at 21–22°C with 12h light and dark cycles. Food (Purina Mouse Chow) and tap water were given *ad libitum*. None of the mice was found to have gross pathological lesions.

Tissue preparations

The protocol for each daily experiment included one mature (17 or 23 months) and one old animal (25, 28 or 31 months). Each mouse was killed by cervical dislocation, the head removed, and the animal exsanguinated. To minimize possible diurnal variation the mice were routinely killed between 8:00 and 9:00h. Liver, kidneys and heart were excised and immediately rinsed in ice-cold 0.85% (w/v) NaCl solution. The chilled organs were trimmed and quickly weighed. All subsequent processing procedures were carried out at 0–4°C. Homogenates (9%, w/v) were prepared in 5.0% (w/v) metaphosphoric acid by using an all-glass Ten-Broeck homogenizer, and 2.0ml portions were centrifuged at 14000*g* for 15min in a Lourdes LRA centrifuge with a 9RA rotor. For the determination of total glutathione (GSH + GSSG), a portion of supernatant was diluted with 0.1M-sodium phosphate/0.005M-EDTA, pH7.5, to lower the concentration of metaphosphoric acid before assay. For the determination of GSSG an equal volume of 0.04M-*N*-ethylmaleimide solution was added to interact with GSH, and the mixture was incubated for 60min at 20–22°C. Excess *N*-ethylmaleimide was removed by extracting eight times with equal volumes of ether, and residual ether was removed by bubbling air through the samples for 20–30min.

Glutathione assay

Total glutathione and GSSG were assayed by a modification of the enzymic cycling method of Tietze (1969) and Owens & Belcher (1965), in which the rate of 5,5′-dithiobis-(2-nitrobenzoic acid) reduction is proportional to the amount of either GSH or GSSG present. Tissue extracts (volume 25–100μl) were added to an assay medium containing 0.5 unit (one unit will reduce 1.0μmol of GSSG/min at pH7.6 at 25°C) of yeast glutathione reductase, 0.5μmol of 5,5′-dithiobis-(2-nitrobenzoic acid) and 0.1M-sodium phosphate/0.005M-EDTA buffer, pH7.5, in a volume of 0.90ml. This reaction mixture was preincubated for 2min at 20–22°C to allow thiol components in the tissue extract to interact with 5,5′-dithiobis-(2-nitrobenzoic acid). Then the enzymic cycling reaction was initiated by the addition of 0.2mmol of NADPH, and the rate of 5,5′-dithiobis-(2-nitrobenzoic acid) reduction was determined from the increase in A_{412} by using a Zeiss PMQ II spectrophotometer. This rate was corrected for the reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) with glutathione reductase alone without any sample. Each sample was analysed at two or more concentrations, and under these conditions the initial rates were proportional to sample size for all tissues. Also, every daily assay included standard curves with known amounts of GSH and GSSG. GSH was calculated from the difference between the total glutathione and GSSG values.

Chemicals

NADPH of 98% purity, yeast glutathione reductase [NAD(P)H–GSSG oxidoreductase, EC 1.6.4.2] (200units/ml), GSH, GSSG, 5,5′-dithiobis-(2-nitrobenzoic acid) and *N*-ethylmaleimide were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and solutions of these reagents were prepared in 0.1M-sodium phosphate/EDTA buffer, pH7.5. Other chemicals were of reagent grade.

Statistical methods

The results were analysed statistically by standard methods described by Snedecor (1946) and by use of a Hewlett–Packard minicomputer.

Results

Validation of the method

An important aspect of this investigation was the use of a specific analytical method for both GSH and GSSG. The enzymic cycling method of Tietze (1969) was selected and evaluated for mouse tissues by determining the optimal assay conditions and by recovery experiments.

To increase the sensitivity of this method the enzyme concentration was decreased from 1 to 0.5unit/ml of assay mixture, and the 5,5′-dithiobis-(2-nitrobenzoic acid) concentration was decreased from 0.6 to 0.5mol/ml. These modifications doubled the sensitivity of the method by lowering the blank rate. The rate of increase in A_{412} was the same for either GSH or GSSG, and the proportional range was 4–400ng of glutathione.

The initial rates and time courses of the enzymic reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by different tissues are shown in Fig. 1. For the measurement of either total glutathione or GSSG in liver, kidney or heart tissues, the increase in A_{412} was proportional to time for the initial 2.5min, with a gradual decrease in rate thereafter. Therefore, for routine assays the initial rates were determined within the first 2min. Also several concentrations of each sample were assayed routinely to ensure proportionality and to detect any endogenous inhibitors or activators.

Recovery experiments were carried out by adding known amounts of GSH or GSSG to the tissue samples before homogenization (Table 1). The average recoveries of GSH in liver, kidney and heart were 101, 96 and 102% respectively, and the

recoveries of GSSG were 105, 104 and 96% respectively. These results established the validity of this overall procedure for the quantitative measurement of GSH or GSSG.

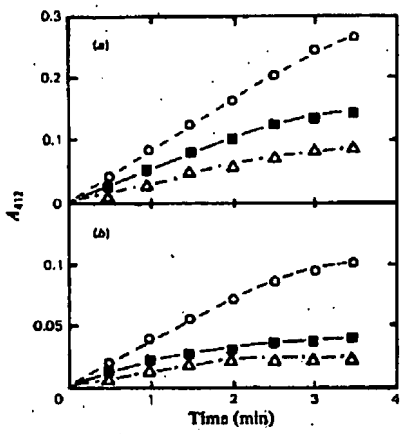

Fig. 1. *Time course of enzymic reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) by different tissues from a 25-month-old mouse*
The rates of 5,5'-dithiobis-(2-nitrobenzoic acid) reduction by tissue samples with or without *N*-ethylmaleimide treatment were analysed for (*a*) total glutathione and (*b*) GSSG as described in the text. The tissues were: O, liver; ■, kidney; △, heart.

Characterization of the experimental organism

Another key aspect of this study was the use of animals of known biological ages as indicated by life-table or survival-curve criteria. Fig. 2 shows the survival curve for C57BL/6J mice in our aging colony (University of Louisville, lot 3/75), which is representative of the other two lots in this investigation (6/76, 9/75). The median survival time was 29 months, a value that has been obtained consistently since 1962 (Lang *et al.*, 1973). Animals in the 75–25%-survival group were defined as biologically old, and those in the 25–0%-survival group

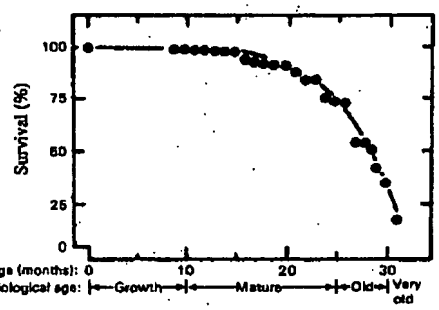

Fig. 2. *Survival of the C57BL/6J mouse*
Data are expressed as percentage survival of a population of 100 male mice (lot no. 3/75). The median survival time (ST_{50}) was 28.9 months.

Table 1. *Recovery of glutathione added to mouse tissue samples*
Known amounts of GSH or GSSG were added to different tissue samples before any tissue processing. The results are expressed as percentages of added glutathione recovered compared with the calculated amounts. The methods used are described in the text.

Tissue	Expt. no.	GSH or GSSG (μequiv. of GSH/g of tissue)	GSH (μg added)	GSH (Recovery, %)	GSSG (μg added)	GSSG (Recovery, %)
Liver	1	6.58 (GSH)	100	94.6	—	—
			200	89.8	—	—
	2	7.29 (GSH)	100	112	—	—
			200	110	—	—
	3	8.02 (GSH)	100	102	—	—
			200	100	—	—
	4	0.208 (GSSG)	—	—	40	107
			—	—	80	103
Kidney	1	2.68 (GSH)	40	99.3	—	—
			80	94.5	—	—
	2	2.59 (GSH)	40	91.5	—	—
			80	96.5	—	—
	3	0.0158 (GSSG)	—	—	10	104
			—	—	20	104
Heart	1	0.876 (GSH)	40	109	—	—
			80	95.5	—	—
	2	0.0187 (GSSG)	—	—	10	97.0
			—	—	20	95.0

as 'very old'. In this study, therefore, the biological and chronological ages of the mice were classified as follows: growing (6 months), mature (17 and 23 months), old (25 and 28 months) and very old (31 months).

The general nutritional status of the animals was determined from the body and organ weights (Fig. 3). As expected for growing animals, increases in both body and organ weights were observed after 6 months of age, which emphasizes a seldom recognized fact that a 6-month-old mouse is still growing, even though sexually mature. From 10 to 36 months the body and organ weights did not change, for the slopes of their age profiles ranged from −0.040 to +0.0096 ($P < 0.2$). Thus by these weight criteria the nutritional status of the mice was adequate and did not change.

Glutathione concentration

The glutathione concentrations in tissues of mice of different ages are shown in Table 2. Comparison of the tissues indicated that the total glutathione content in liver was about 3 times that in kidney and 10 times that in heart at all ages. Over 97% of the total glutathione was present as GSH, and GSSG comprised only a small percentage of the total: 2.6, 0.65 and 1.75% for liver, kidney and heart respectively.

In liver, two distinct age-related decreases occurred in GSH content, but GSSG concentrations were

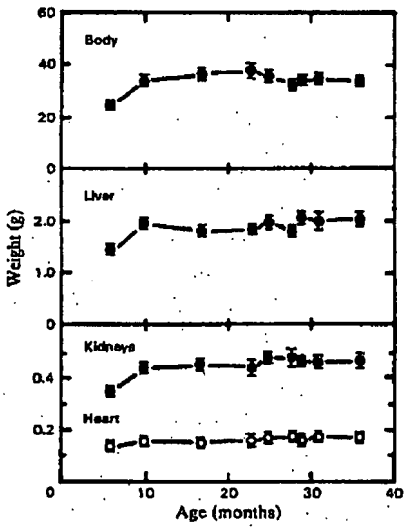

Fig. 3. *Body and organ weights of C57BL/6J mice during their life-span*
The body weights, liver weights, paired kidney weights and heart weights were determined from four different cohort populations of mice studied over a period of 18 months. Each point and bar represents the mean ± S.E.M. of three to ten mice. The methods used are described in the text.

Table 2. *Glutathione concentrations in tissues of C57BL/6J mice during their life-span*
The results are expressed as means ± S.E.M. for the indicated numbers of mice. The methods used are described in the text.

Tissue	Age (months)	No. of mice	Glutathione (μequiv. of GSH/g of tissue) GSH	GSSG	Total
Liver	6	5	8.57 ± 0.318*	0.167 ± 0.0304	8.73 ± 0.302
	17	4	7.85 ± 0.332	0.219 ± 0.0329	8.05 ± 0.352
	23	3	7.79 ± 0.358	0.212 ± 0.0202	7.98 ± 0.342
	25	4	7.39 ± 0.710	0.192 ± 0.0282	7.59 ± 0.736
	28	4	6.78 ± 0.388*	0.187 ± 0.00857	6.97 ± 0.388
	31	6	5.47 ± 0.274**	0.181 ± 0.0143	5.67 ± 0.284
Kidney	6	5	3.32 ± 0.119*	0.00781 ± 0.00302	3.32 ± 0.123
	17	4	2.73 ± 0.217	0.0203 ± 0.00308	2.75 ± 0.218
	23	3	2.69 ± 0.170	0.0184 ± 0.00217	2.71 ± 0.170
	25	4	2.66 ± 0.309	0.0147 ± 0.00671	2.67 ± 0.316
	28	4	2.19 ± 0.119*	0.0228 ± 0.00297	2.21 ± 0.120
	31	6	1.80 ± 0.0550**	0.0130 ± 0.00315	1.81 ± 0.0570
Heart	6	5	0.928 ± 0.0420**	0.0117 ± 0.00301	0.941 ± 0.0420
	17	4	0.821 ± 0.0326	0.0187 ± 0.00544	0.837 ± 0.0456
	23	3	0.730 ± 0.0892	0.0130 ± 0.00423	0.743 ± 0.0896
	25	4	0.720 ± 0.0573	0.00814 ± 0.00282	0.726 ± 0.0586
	28	4	0.805 ± 0.0183	0.0187 ± 0.00450	0.827 ± 0.0192
	31	6	0.651 ± 0.0361*	0.0108 ± 0.00287	0.664 ± 0.0365

* $P < 0.05$ and ** $P < 0.001$ when compared with the pooled values for 17- and 23-month-old mice.

constant. In the 6-month-old mouse there was a high GSH content, which decreased and reached a plateau by 17 months, reflecting a change from growth to maturation. Later there was a second decrease after 23 months of age, which represented an aging or senescent change.

In kidney and heart tissues similar decreases in GSH concentrations with age were observed, as well as unchanged GSSG content. However, in heart tissue, the GSH decrease occurred later (after 28 months) than in liver or kidney.

The changes are illustrated more clearly by the life-span profiles of relative GSH concentrations (Fig. 4). The results were expressed relative to values for the 17-month-old biologically mature group. At earlier ages growth-related decreases of 9, 21 and 13% were observed in liver, kidney and heart respectively. Of special importance were the aging-specific decreases in GSH concentration, which occurred between mature and very old mice: 30% in liver, 34% in kidney and 20% in heart. These changes were considered aging-specific, since they coincided with the rapid decrease in survivors occurring at that time, and thus the GSH profile resembles the survival curve (Fig. 2).

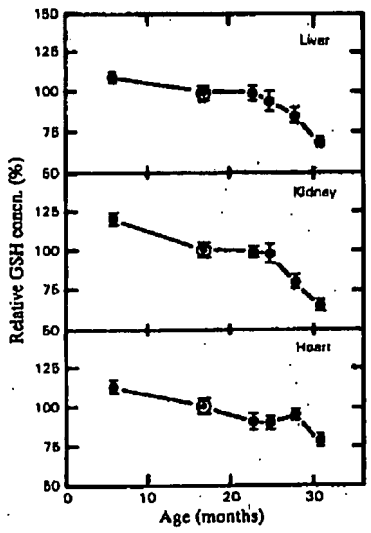

Fig. 4. *GSH contents of tissues during the life-span of the mouse*

GSH concentrations of liver, kidney and heart tissues were determined as described in the text. The GSH concentrations were expressed relative to the values at 17 months as 100% given in Table 2. Each point and bar is the mean ± S.E.M. of three to six mice.

Discussion

The present evidence indicates that a low GSH content may be a general phenomenon of all aging tissues and not restricted to a few specialized tissues. Significant decreases of 20–34% in GSH and total glutathione concentrations were observed and were aging-specific, since they coincide with senescence according to survival curves. In addition to mammalian tissues, a similar decrease in GSH contents occurs in senescent insects (Hazelton & Lang, 1978*b*).

In the present study the observed decreases in GSH concentration were independent of changes in tissue composition, because body and organ weights were unchanged during maturity and senescence. Also, other parameters such as dry weight and water, lipid, protein and DNA contents were constant during these periods (G. A. Hazelton & C. A. Lang, unpublished work).

The findings are consistent with our previous results of aging-specific decreases in glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities, which led to our hypothesis that a decreased reducing potential occurs in senescent tissues. Indeed GSH may be the key factor in lowering this potential. In support of this is the evidence that cellular GSH concentration may have a profound regulatory effect on the activity of pentose phosphate-cycle enzymes (Hosoda & Nakamura, 1970; Hochstein & Utley, 1968; Jacob & Jandl, 1966). Owing to decreased GSH concentrations, the $NADP^+$ coenzyme that is generated by the coupled enzyme system of glutathione (GSSG) reductase and glutathione (GSH) peroxidase may become limited and less available to the pentose phosphate-cycle enzymes. As a result, NADPH reducing equivalents may decrease and in turn cause a lower biosynthetic activity.

Decreased GSH concentrations could have a marked effect on the detoxification capacity of a senescent organism, since a major function of GSH is in the detoxification of peroxides produced by normal metabolism and of xenobiotics via glutathione *S*-transferases. Thus this lower capacity may provide a toxicological basis for aging.

Our results also indicate that growth-specific changes in GSH tissue contents occur between 6-month-old and 17-month-old mice. These findings are similar to the decreases observed in liver GSH concentrations between 3- and 18-month-old rats (Bartoc *et al.*, 1975). However, in this case the changes were incorrectly called aging rather than growth changes. Indeed, a 3-month-old rat is still growing and an 18-month-old rat is mature but not senescent (Berg & Simms, 1960).

We found low GSSG concentrations in the mouse, as others have in the rat (Wendell, 1970; Tietze, 1969). Also, the GSSG contents of tissues

were unchanged during the periods of growth, maturity and senescence. This is in contrast with the results of Oeriu (1964) and Oeriu & Tigheciu (1964), who reported that GSSG concentrations increased with age and were 7–8 times higher than our values. Since no methodological details or recovery experiments were presented, it is uncertain whether their results were due to aging or to autoxidation. Indeed a possible explanation for this discrepancy is that their method may overestimate GSSG. In this regard Wendell (1970) has pointed out the shortcomings of measuring GSSG by difference in the amount of GSH assayed before and after chemical reduction. Thus autoxidation of even a small proportion of the GSH can appear as a large increase in GSSG content.

Several possibilities exist for the occurrence of lower GSH concentrations in aging tissues. One is that the loss of GSH is a result of enhanced oxidation in the old mouse. However, the low and constant GSSG concentrations at different ages indicate otherwise. Another possibility is that the lower GSH content may be a result of either decreased synthesis or increased degradation of total glutathione. It is of special interest that erythrocytes of sheep with glutathione deficiency also have a lower γ-glutamylcysteine synthetase activity, which is required for the first step of glutathione biosynthesis (Smith *et al.*, 1973).

A third possibility for the lower GSH concentrations is an increased utilization of GSH in the removal of peroxides and the detoxification of foreign compounds. Thus it is conceivable that there may be an accumulation of toxic substances with aging, which would increase glutathione peroxidase and transferase activities and eventually deplete the intracellular GSH pool. Direct evidence to choose among these possible mechanisms will require further investigation.

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